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EFFICACY OF HORMONE-TOXIN FUSION PROTEIN IN CELL CULTURE

By

MORGAN DILLARD

A Thesis Submitted to the University Honors Program in Partial Fulfillment of the Requirements
for the Honors Degree

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Abstract

Luteinizing hormone (LH) and human chorionic gonadotropin (hCG) are members of the gonadotropin family and are vital for fertility in animals. Both LH and hCG bind to the luteinizing hormone receptor (LHR), found only in gonadal cells such as the Leydig cells of the testis and the granulosa and theca cells of the ovary. Binding of the hormone to its receptor causes production of the steroid hormones testosterone and estrogen. By ablating cells that express LHR, the production of sex steroids would be terminated, leading to a loss in fertility. The overall goal of the project is to create a nonsurgical, single-dose sterilant for cats and dogs by ablating these cells. One way to target these cells is the creation of a fusion protein that includes a toxin targeted to these cell types. This paper will focus on determining the efficiency in production of these fusion proteins in cell culture. Diphtheria toxin (DT) and *Pseudomonas* endotoxin A (PE) are both suitable toxins known to be successfully inserted into fusion proteins. By replacing the receptor-binding domain in these toxins with hCG, the toxin will be ineffective in all cells except those expressing LHR. To test how much fusion protein is produced in cells, Chinese hamster ovary (CHO) cells and alpha mouse liver (AML) cells were used to produce fusion proteins. While the total protein concentration was higher in the transfection media of AML cells compared to CHO cells, no hCG was detected in the AML transfection media. Therefore, AML cells may not produce sufficient amounts of the fusion protein to quantify. CHO cells were used to produce six fusion proteins using both DT and PE. The fusion proteins that contain DT were expressed at lower concentrations than yhCG alone, while the fusion proteins containing PE could not be detected at all in the CHO media. These results suggest that the addition of the toxin to hCG decreases either transfection and/or production efficiency and that PE may have an adverse effect on cell health, which would lead to poor expression.

Acknowledgement

I would like to thank Dr. Prema Narayan, for the support and guidance she has given me during my time in her lab. I would also like to thank Stacey McGee and the rest of the Narayan lab for their help and support as I completed this project.

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Introduction

Luteinizing Hormone, the Luteinizing Hormone Receptor, and Human Chorionic Gonadotropin

The anterior pituitary gland secretes two gonadotropins: luteinizing hormone (LH) and follicle stimulating hormone (FSH). These hormones are produced starting at puberty and act on the gonads of both sexes. LH binds to the luteinizing hormone receptor (LHR), found only in theca and granulosa cells of the ovaries and Leydig cells in the testes. LH is necessary for the production of steroid hormones such as testosterone and estrogen (1). LH is required for both spermatogenesis and ovulation. If LHR were eliminated, there would be a reduction and most likely termination of the production of sex hormones. This lack of hormones would then cause infertility, due to the lack of mature sperm and eggs.

Human chorionic gonadotropin (hCG) is another gonadotropin; however, hCG is synthesized in the placenta of primates and equines, not in the anterior pituitary gland. All three of the gonadotropins share similar structures, in that they are all glycosylated heterodimers (2). All gonadotropins share a common alpha subunit while the beta subunits are specific to each hormone. The hCG beta subunit contains a unique addition to the C-terminus in the form of a 30 amino acid residue extension (3). It has been demonstrated that this can increase the half-life of this hormone *in vivo*, as there is no role for it in the activity of the hormone (4,5). Glycosylated hCG has a total molecular weight of 38,000 kDa. The amino acid sequence for hCG shares a high amount of homology with the sequence for LH (2). This leads to the ability for hCG to bind to LHR with an even higher affinity than LH.

While hCG has a heterodimeric structure, it was shown that a single chain homolog, in which the alpha and beta subunits are synthesized together, has the same binding affinity to LHR

as native hCG. This single chain or yoked hCG (yhCG) is used in this experiment to ensure that both the alpha and beta subunits are produced by the cells (6).

hCG-Toxin Fusion Proteins for Cell Ablation

Immunotoxins are modified protein toxins that are targeted to act on a certain type of cell. This specificity comes from the replacement of the native receptor-binding domain with an antibody or hormone that targets one type of cell. The toxin can then be internalized by that cell type and act on it, leaving cells without the specific receptor unharmed. This method of cell targeting has been used in clinical trials for targeting different types of tumor cells as a possible cure for cancer (7). In this project, the receptor-binding of the toxins are replaced with yhCG, so that the toxins will only be able to act on cells that contain LHR. Therefore, the toxin action will be targeted to gonadal cells expressing LHR and halting the production of sex steroids. This will in turn cause infertility due to lack of mature gametes.

Two toxins are used in this project to create fusion protein constructs and to test in cell culture. The two toxins are DT and PE, and these were chosen because of two reasons. The first reason is that while both toxins contain a catalytic (C) domain, a translocation (T) domain, and a receptor-binding (R) domain, the orders of these domains are different between the toxins. In DT, the R domain is found at the C terminus of the sequence (8), while the R domain of PE is found at the N-terminus of the sequence (9,10). There is a possibility that the difference in R domain location could lead to a difference in binding of the toxin to the cell receptor. By testing both of the toxins, the potential difference can be accounted for.

Another reason for choosing the two toxins is the difference in their intracellular trafficking. DT attaches to the target cell via the R domain, where the toxin is cleaved between the C and T domains, leaving a disulfide bond connecting the two domains. The cleaved toxin is

brought into the cell through clathrin-coated pits, which mature into early endosomes. A channel is formed in the endosome by the T domain, which causes the C domain to translocate across the membrane. When in the cytoplasm, the catalytic domain is able to inhibit protein synthesis by the ribosylation of an elongation factor (eEF-2), resulting in cell death (11) (Figure 1). DT has such a high level of cytotoxicity that one molecule of the C domain has been found to be adequate to kill the cell (12).

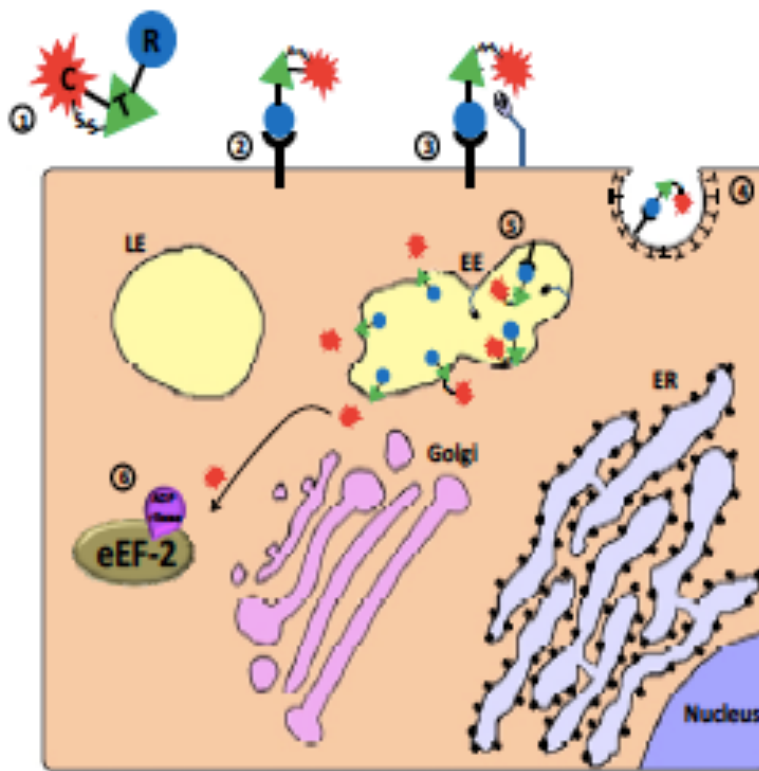


Figure 1. Mode of action of DT. Adapted from reference 7. 1. DT before binding to the cell. C and T domain connected by an extra disulfide bond. 2. R domain binds to the cell surface receptor. 3. The bond between the C and T domains is cleaved, leaving only a disulfide bond connecting them. 4. The toxin is internalized through clathrin-coated pits, which turn into early endosomes. 5. In the early endosome, the T domain creates a channel in the membrane and the C domain is translocated into the cytoplasm. 6. The C domain inhibits eukaryotic elongation factor-2 (eEF2) through ADP ribosylation to cause cell death by halting protein synthesis.

PE is also internalized by clathrin-coated pits after binding to its cell surface receptor and the terminal lysine is removed from the REDLK signal sequence. In the early endosome, the toxin is cleaved to yield an active fragment containing the C (III) domain and two-thirds of the T (II) domain. This fragment is transported through the trans-Golgi network, where the REDL sequence binds to the KDEL receptor and is sent to the endoplasmic reticulum. Sequences in domain II cause the retro-translocation to the cytoplasm, when the catalytic domain targets and inhibits eEF2 to cause cell death. (7) (Figure 2).

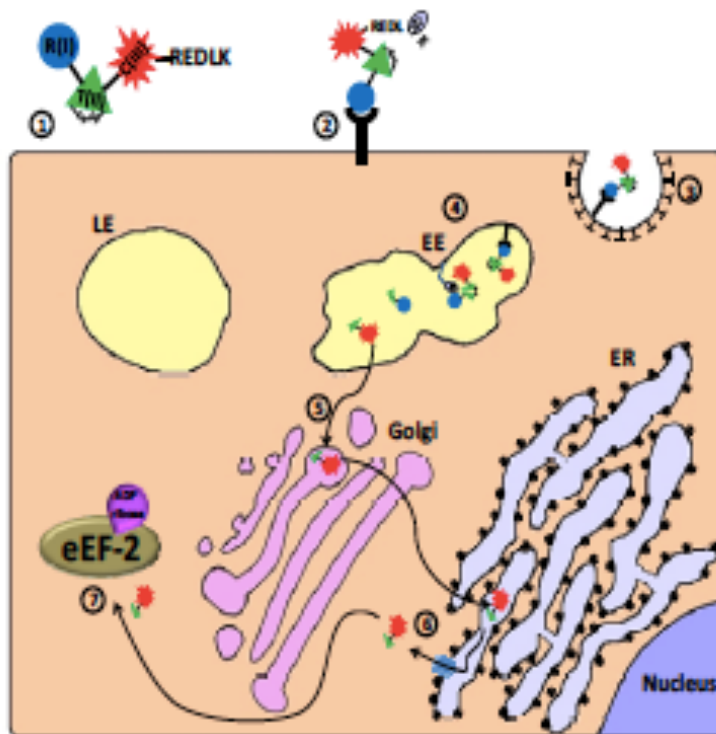


Figure 2. Mode of action of PE adapted from reference 7. 1. PE sequence before binding to the receptor. 2. The terminal lysine is removed from the signal sequence and the toxin binds to the cell receptor through domain I. 3. The toxin is internalized via clathrin-coated pits. 4. The C domain and two-thirds of the T domain are cleaved from the rest of the toxin. 5. The active fragment is transported to the trans-Golgi network. 6. The fragment is routed to the ER through the REDL signal sequence and is retro-translocated to the cytoplasm. 7. PE targets eEF2 to inhibit protein synthesis and cause cell death.

Six toxin constructs were created (Figure 3) so that testing can be done to determine which immunotoxin has the best activity. DT and PE have both been previously used to generate fusion proteins for targeting tumors expressing different receptors (7,13,14,15). Other differences between the constructs include the presence of an additional CTP sequence between the toxin and the yhCG in order to allow for more flexibility and possibly better folding. Also, in the PE constructs, the substitution of a KDEL sequence in the place of the native REDLK sequence will be created. This replacement has been shown to increase PE's cytotoxicity, which is why it is of interest (15).

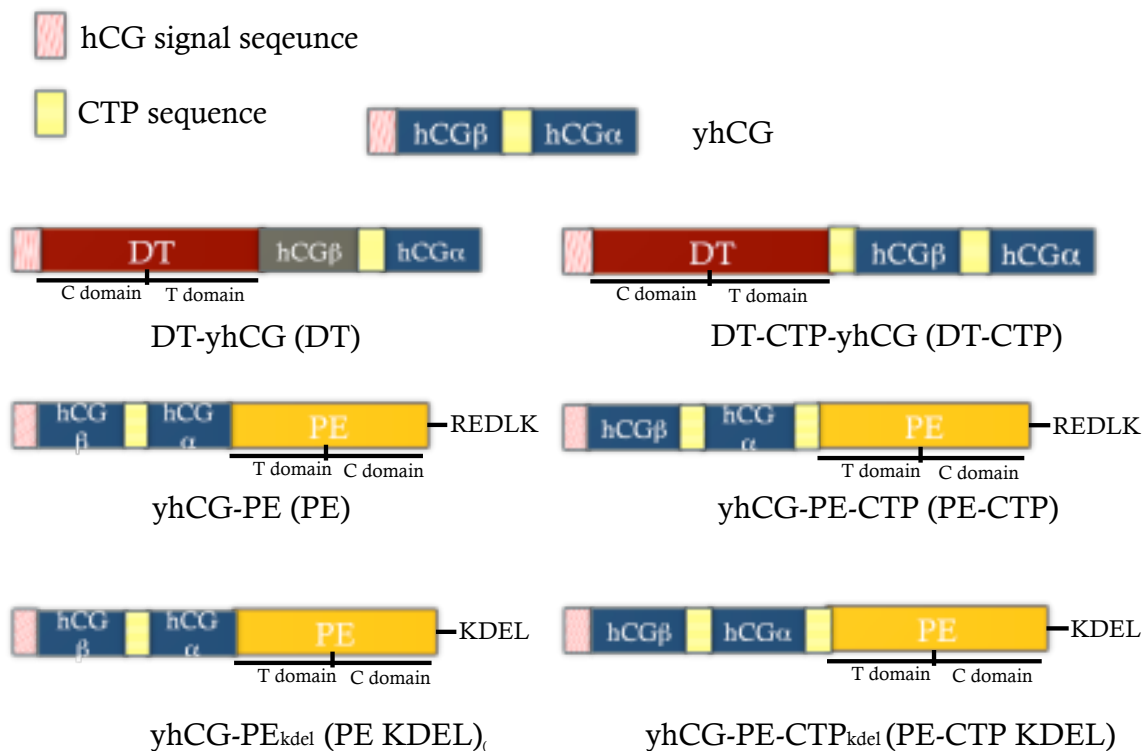


Figure 1. Schematic of hCG-toxin fusion proteins. These constructs were generated by Stacey McGee in the Narayan lab.

CHO and AML Cell Lines

CHO cells are a commonly used cell type in biological laboratories because they are easy to maintain and produce large amounts of protein (16). Therefore, they are an ideal cell line to use in this project to produce the fusion proteins. These cells are used as a control line because of their known protein production. To assist in discovering the effects of these fusion proteins on target tissues, AML12 cells will also be used as a production cell line. In the final product, the fusion toxin construct will be targeted to the liver, for protein expression and secretion into circulation. Therefore, the potential effects of the toxin on the liver are of interest, as well as determining whether these liver cells are capable of producing the toxin.

Goal

The goal of the project is to create a nonsurgical, single-dose sterilant for cats and dogs. The specific goal of my portion of the project is to determine the amount of toxin fusion proteins produced by AML and CHO cells. Before testing these fusion proteins in murine animals, they must be tested in cell culture to determine their effects. The amount of fusion protein produced by the cells may vary with cell type, so two different lines are used. AML are used to produce the fusion proteins because liver cells will be targeted in the *in vivo* studies. CHO cells are used as a control because they are known to produce large amounts of proteins.

Experimental Methods

Cell lines, media, and culture conditions

CHO and AML12 cells were purchased from American Type Tissue Culture. CHO cells were maintained in DMEM/F12 media with 10% fetal bovine serum at 37°C with 5% CO₂. AML12 cells were maintained in DMEM/F12 media with 10% fetal bovine serum and dexamethasone at 37°C with 5% CO₂.

*Transient transfection of *yhCG* and fusion proteins*

In order to transiently transfect cell lines with desired plasmids, Lipofectamine 3000 transfection reagents were used. Cells were grown in 10 cm dishes until 70-80% confluency in their appropriate media. Just before transfection, the serum-containing media was replaced with serum-free media. 750 µl of Optimem Reduced Serum Media was mixed with 15ug of construct DNA and 30 µl of P3000 reagent. A separate solution of 750 µl Optimem and 22.5 µl Lipofectamine reagent was also prepared. These two solutions were mixed and allowed to sit for 5 minutes. Then the mixture was pipetted onto the cell plates and allowed to incubate for 5 hours. The media was removed and replaced with complete, serum-containing media, and the cells were incubated overnight. Then, the complete media was replaced with serum-free media

and the cells were incubated for 48 hours. After 48 hours, the serum-free media was collected. Cells were transfected with yhCG or one of the following fusion toxin constructs: DT-yhCG, DT-CTP-yhCG, yhCG-PE, yhCG-PE-CTP, yhCG-PE KDEL, and yhCG-PE-CTP KDEL.

Concentration of Transfection Media

To concentrate the serum-free media collected from the transfection, first, 500 μ l were aliquoted to store as an unconcentrated source. The rest of the media (9.5mL) was loaded into Vivaspin 6 centrifugal concentrators (GE Healthcare). For the yhCG-transfected cells, concentrators with a 10 kDa MWCO were used while concentrators with a 30 kDa MWCO were used for the toxin constructs. The concentrators were centrifuged at 10,000 x G for 30 minutes, or until the media had been concentrated to 200 μ l. The media was aliquoted and stored at -20°C.

For one sample of the AML media, a portion was concentrated additionally using a vacuum concentrator to ensure a detectable amount of protein was in the sample. The media was centrifuged for 30 minutes, then collected.

Protein Assay

The total protein concentration of the media was assayed using a BioRad protein assay. 10 μ l of BSA standards and concentrated transfection media was added to wells on a 96 well assay plate. 200 μ l of BioRad dye reagent (1:5 in water) was added to the samples. The plate was incubated at room temperature for 5 minutes and read at 595 nm.

Western blot analysis of toxin expression

The standard protocol of western blot analysis was used to detect expression of the toxins (17). A 10% Laemmli discontinuous gel was used. On the gel, 30 μ l samples of concentrated media from transient transfections of AML and CHO cells were loaded into the wells. The

proteins were transferred to a PVDF membrane. A CTP antibody was used to detect yhCG and the fusion proteins.

ELISA analysis of total hCG production

An ELISA kit (Sigma-Aldrich, SE120063) was used to quantify the amount of hCG in the transfected media. hCG MAb coated wells were filled with 50 μ l of hCG standards, control and samples of CHO and AML media from transient transfections of yhCG and the fusion proteins. Then 100 μ l of enzyme conjugate was added to all wells. The plate was covered and incubated with gentle agitation for 1 hour at room temperature. After 1 hour, the liquid was removed from the wells, which were rinsed. 100 μ l of TMB substrate was added and incubated for 10 minutes. Then 50 μ l of stop solution were added to the wells and mixed. The wells were read at 450nm.

Results and Discussion

Because CHO cells are known to be reliable producers of proteins, both AML and CHO cells were transfected with yhCG to determine the efficiency of protein production in AML cells. The protein assay shown in Figure 4 indicates that the AML media had a higher total protein content than the CHO cells. However, the protein assay is not specific for the target protein, yhCG. Therefore, western blot analysis using a CTP antibody specific for hCG β was performed to quantify the relative amounts of yhCG that had been produced by the cells. The results of the western blot showed bands in both the hCG control and CHO transfection media lanes, but not in the AML transfection media lanes (Figure 5). The difference in band size can be due to difference in glycosylation in the samples, and because in the hCG lane, the α and β subunits are

separated. Therefore, it was determined that although the AML cells had a higher total protein concentration, the transfection was not successful enough to give a detectable amount of yhCG.

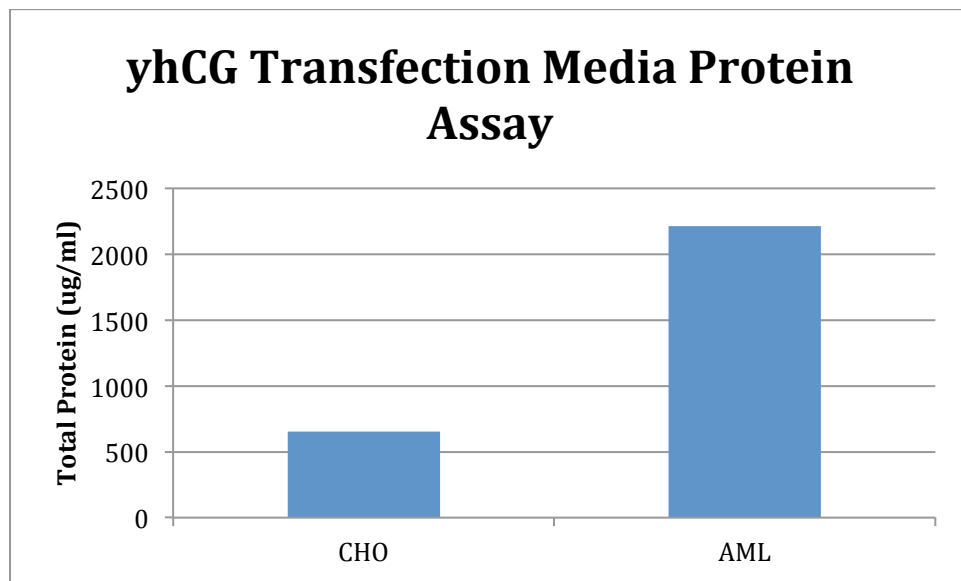


Figure 4. Total protein concentration in media of CHO and AML cells transfected with yhCG. Media was concentrated and assayed for total protein.

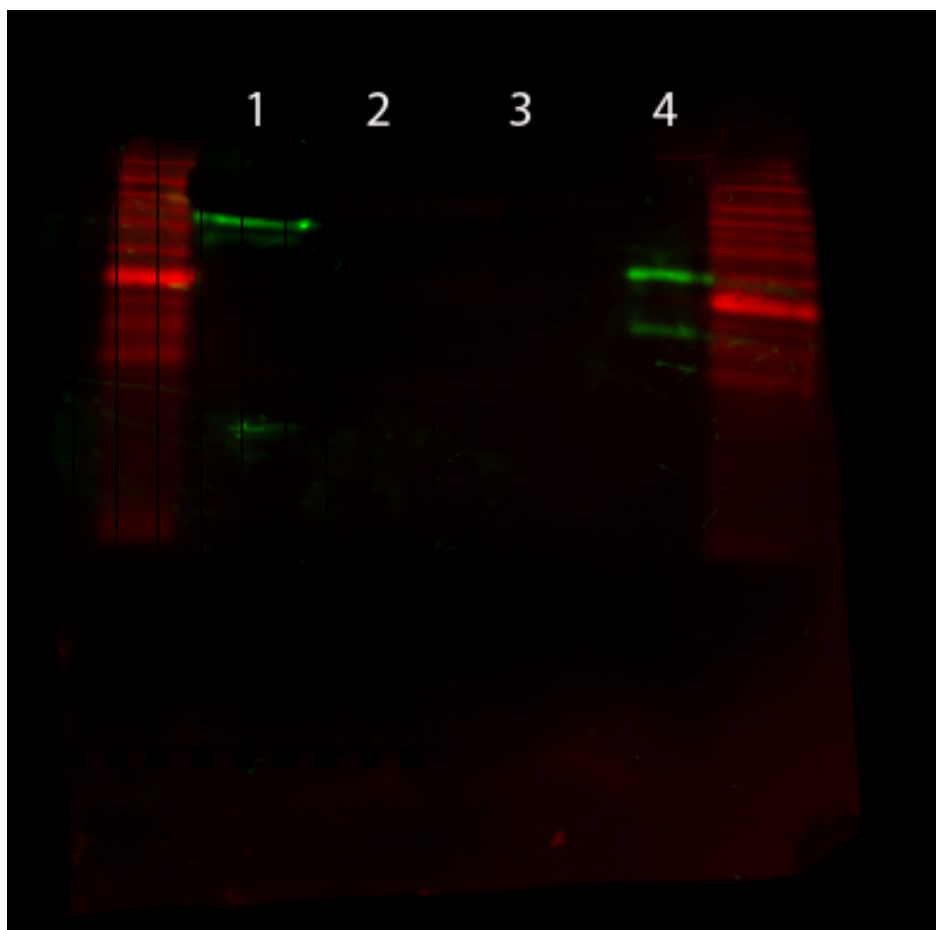


Figure 5. Western blot analysis of CHO and AML yhCG transfection medias with CTP antibody. Lane 1 contains 30ng of pure hCG. Lane 2 contains AML transfection media concentrated using column concentrators (10.4 μ g total protein). Lane 3 contains AML transfection media concentrated using column concentrators and vacuum concentration (54 μ g total protein). Lane 4 contains column concentrated CHO transfection media (26.1 μ g total protein).

Because of the findings of the western blot analysis, CHO cells were used for the rest of the project to produce the fusion proteins. Transient transfections with each construct were performed in CHO cells in order to have sufficient protein for analysis. Next, the total protein content of each sample of concentrated transfection media was determined (Figure 6). The transfections with the different constructs produced a similar amount of total protein, with both DT and PE constructs producing between 4000 and 5000 mg/ml of total protein. We would

expect that the amount of yhCG/fusion protein produced should be proportional to the total protein concentration. To determine this, an ELISA was performed that is specific for hCG.

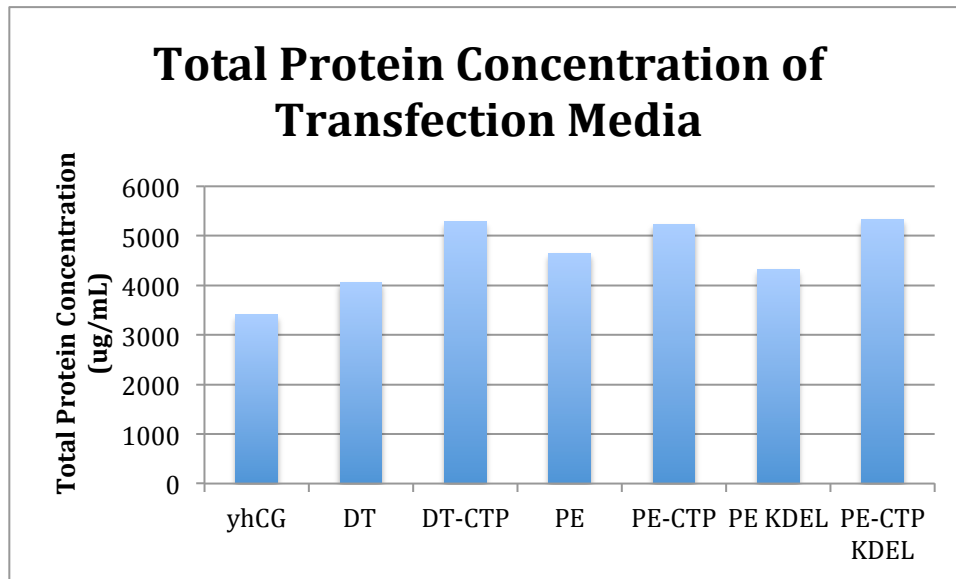


Figure 6. Total protein concentration in CHO transfection media. The media was collected, and concentrated, then assayed to determine protein production.

Because all of the constructs contain yhCG as the R domain, the ELISA should be able to detect all of the constructs, no matter what toxin was attached. The results for the ELISA can be found in Figure 7. Surprisingly, only the yhCG, DT, and DT CTP produced detectable levels of hCG. All four PE constructs resulted in levels of hCG-toxin that was below the limit of detection of the ELISA. Even the DT constructs had a much lower concentration of hCG than the yhCG, which suggests that either the addition of the toxin to the protein may inhibit synthesis in some way or that the fusion with the toxin prevents the antibody used in the ELISA from detecting hCG. This data may suggest that PE has a negative effect on the cells when produced, which would lead to lower fusion protein expression.

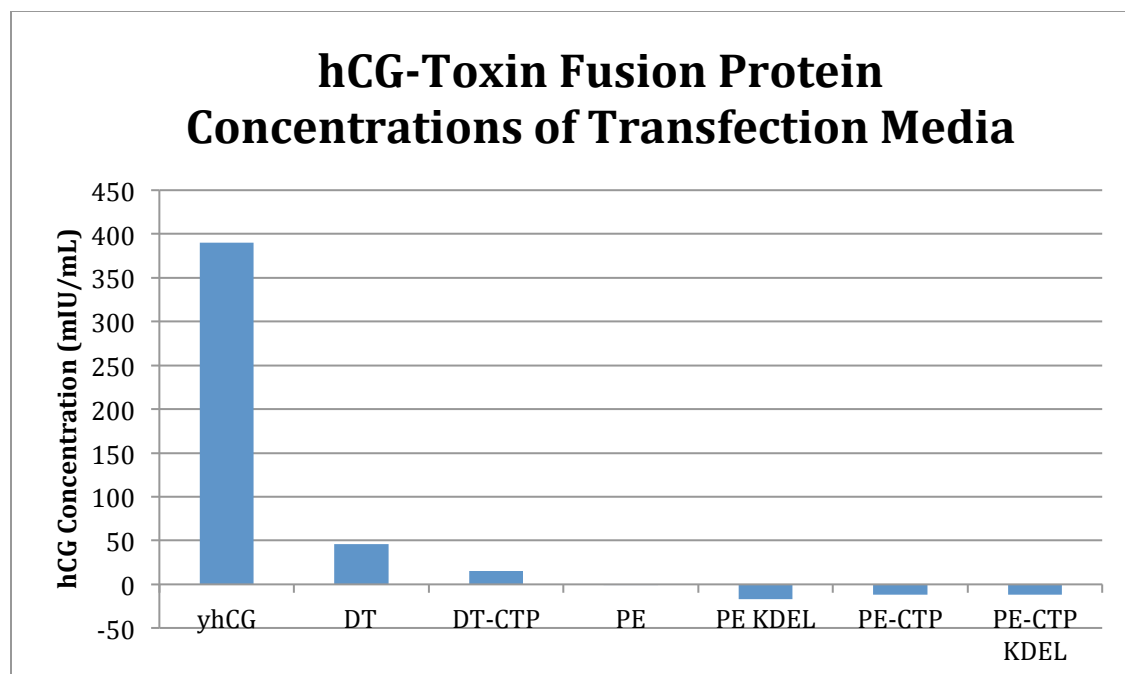


Figure 7. Concentration of hCG in media from CHO cells transfected with the various constructs. Media was collected, concentrated and the concentration of yhCG or fusion proteins was measured using a hCG specific ELISA kit.

This project determined that CHO cells can be used to produce hCG-toxin fusion proteins in cell culture, while AML cells were not able to produce a detectable amount of the protein. However, when the amount of fusion protein was quantified using ELISA, the fusion proteins containing DT were present in a much lower amount than yhCG, while those containing PE were not detectable in the cell media at all.

Future studies will be done to optimize the transfection conditions for CHO and AML cells to increase the efficiency of fusion protein production. Once the fusion proteins are produced, binding assays will be performed to determine whether or not these fusion proteins can target the cells that express LHR. This will also determine if the fusion proteins have the same affinity for LHR as native hCG. By completing these binding assays, we hope to discover

whether or not the fusion proteins can successfully target and ablate the gonadal cells they are intended to destroy.

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